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Identification, Functional Analysis and Preliminary Validation of Differentially Expressed Genes in Hyperacute Cerebral Infarction Patients

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Key Words

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Abstract

Background/Aims: At present, most studies on ischemic stroke and micro RNA are focused on 24h after stroke. Therefore, our experiment intends to analyze the differentially expressed genes in hyperacute cerebral infarction patients, and to analyze the relationship between the most significantly differentially expressed miRNA and clinical characteristics.

Methods: Download miRNA expression microarray and gene expression microarray from GEO database, screen differentially expressed genes and miRNA by bioinformatics method, and analyze their biological functions. The peripheral plasma of patients with hyperacute stroke was collected, the related miRNAs were extracted, and the relationship between them and clinical characteristics was analyzed.

Results: The microarray included 39 hyperacute cerebral infarction patients and 33 healthy volunteers and 5 differentially expressed miRNAs and 1107 differentially expressed genes were obtained by analysis. MiR-3156-5p was selected to further analyze and verify the correlation with the clinical characteristics of patients. The results showed that the expression level of miR-3156-5p was significantly positively correlated with triglyceride level, and had a significant negative impact on stroke. The low level of miR-3156-5p in hyperacute cerebral infarction patients may be one of the initiating factors of neuroinflammation induced by ischemic stroke, and functional analysis and some existing experimental results also support this view.

Conclusions: In our study, a series of differentially expressed genes and differentially expressed miRNAs were screened by analyzing GEO data sets. In addition, the low expression of miR-3156-5p in hyperacute cerebral infarction patients may be one of the initiating factors of neuroinflammation induced by ischemic stroke.

1. Introduction

Ischemic stroke(IS) refers to ischemic necrosis of localized brain tissue caused by ischemia and hypoxia due to circulatory disturbance of the brain[1]. According to statistics, more than 8 million new cases with IS patients occur in every year, which causes about 6 million 500 thousand deaths, and the number is increasing annually[2,3,4].The specific pathogenesis of stroke is not clear, but it is generally believed that there are a series of pathophysiological processes including oxidative stress, apoptosis, blood-brain barrier destruction, cerebral edema, reperfusion injury and calcium overload. There is evidence that the pathophysiological process of stroke is also affected by genetic and environmental factors[5,6].

Micro RNA(miRNA) is a class of non-coding RNA encoding 20~25 nucleotides[7]. They bind to the untranslated region of target mRNA and play key roles in gene regulation at different stages of cell differentiation and development[8]. MiRNA is involved in almost all biological processes, and is closely related to a variety of diseases, including IS. In addition, miRNAs may be used as a biomarker for diagnosis and prognosis of IS, because they plays a major role in gene control and is stable and detectable in many body fluids including blood[9,10,11]. The role of miRNAs in IS has been extensively studied. MiR-424, miR-124 and miR-let-7c-5p can protect ischemic brain tissue by inhibiting the activation of microglia and the occurrence of neural inflammation after cerebral ischemia[12,13,14]. Overexpression of miR-424, miR-23a-3p and miR-99a can alleviate oxidative stress and protect the brain after ischemic injury[15,16]. Stroke can induce expression of matrix metalloproteinase(MMP), which damage the integrity of endothelial cells and increase the permeability of blood-brain barrier(BBB). MiR-130a inhibitors can significantly reduce encephaledema and permeability of BBB and can improve neurological function by up regulating caveolin-1 and decreasing the level of matrix metalloproteinase-2/9 (MMP-2/9)[17,18]. Studies have shown that over expression of miR-223 can reduce the expression levels of NR2A subunit of NMDA receptors and GluR2 subunit of AMPA receptor (AMPA receptor (AMPA) eventually prevents neuronal death after transient global ischemia[19]. After the onset of ischemic stroke, circulating miR-223 levels increased widely, and were confirmed to be associated with stroke severity and infarct volume in IS patients[20]. Although the available data show that there has been some achievements in the study of the correlation between miRNA and IS, most studies on IS and miRNA are focused on 24h after stroke. Therefore, our experiment intends to analyze the data sets of miRNA and mRNA of hyperacute cerebral infarction(HACI) patients (admission within 6h of onset) in Gene Expression

Omnibus(GEO,<http://www.ncbi.nlm.nih.gov/geo/>) database[21]. The most significantly differentially expressed miRNA after stroke was selected to analyze the relationship between stroke related miRNA and risk factors and examination indexes of stroke patients.

2. Materials and methods

2.1 Patient selection and differential gene screening

MiRNA expression microarray and gene expression microarray were downloaded from the GEO database. The data set meets the following conditions:(1) MiRNA expression microarray and gene microarray containing the whole genome.(2) Stroke patients and normal control group were included. (3) The onset time of stroke was less than 6 hours. In R environment, limma package is used for background correction, standardization and log2 conversion. Subsequently, differentially expressed miRNAs(DEMs) and differentially expressed genes(DEGs) were screened, and the miRNA with the most differential expression was selected for further experimental analysis.

In our study, we collected 58 cases of peripheral blood samples from the Second Affiliated Hospital of Harbin Medical University (including 28 cases of HSCI patients and 20 cases of healthy volunteers(HVT)). The inclusion criteria were as follows: (1)clinical diagnosis of ischemic stroke. (2)Admission within 6h of onset. The exclusion criteria were as follows:(1)having received thrombolytic therapy or currently using anticoagulants. (2)Cerebral hemorrhage, brain trauma and other non ischemic stroke diseases can cause qualitative changes in brain tissue. (3)Patients with transient ischemic attack. (4)Combined with other nervous system diseases or mental diseases. (5)Combined with immune diseases. (6)Combined with tumor.

Collect the basic information of patients, including age, gender, past medical history, and relevant laboratory examination results. The National Institute of Health Stroke Scale(NIHSS) score was performed on admission, and the infarct volume was evaluated by Magnetic Resonance Imaging(MRI). The infarct volume was equal to the sum of the area of each layer of high signal area multiplied by the thickness of each layer.

2.2 Pathway enrichment analysis

To further explore the biological functions and involved pathways of the DEGs, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathway enrichment analysis was performed based on the webGestalt database with the threshold of $P < 0.05$ [22,23].

2.3 Construction and module analysis of PPI network

Protein-protein interaction (PPI) network analysis can deeply understand the functional relationship of gene interaction, and help to systematically study cell function, molecular mechanism of disease, and explore new drug targets. String database and Cytoscape software are used to build PPI network of DEGs. String database (<http://string-db.org>) [24] is a software system for online identification of known protein-protein interactions. The results come from experimental data, text mining and predictive bioinformatics data. In this study, PPI pairs of the DEGs were selected based on the STRING database with the threshold of combined score > 0.4 . The PPI network of the DEGs was constructed based on these pairs.

2.4 Target gene prediction and interaction analysis of DEMs

The target genes of miRNAs were predicted by miRDB [25], TargetScan [26] and DIANA [27] online analysis tools. In order to further improve the reliability of bioinformatics analysis, we use Wayne map to identify overlapping target genes, and further extract the intersection of these overlapping genes and DEGs to obtain key genes.

2.5 RT- qPCR

After centrifugation of peripheral blood samples, plasma was stored in -80°C refrigerator. After the samples were collected, RNA was extracted from plasma samples with BIOZOL reagent and Biospin miRNA extraction kit. MiRcute enhanced miRNA cDNA first strand synthesis kit and miRcute enhanced miRNA fluorescence quantitative detection kit were used for cDNA reverse transcription and RT- qPCR. MiR-423-5p was used as internal reference, and the expression level of related genes was analyzed by $\Delta\Delta t$ method. The primers of miR-423-5p and other miRNAs were designed and synthesized by Harbin Zhongke saines company.

2.6 Relationship between miRNA level and clinical characteristics

The relative expression of miRNA in plasma was determined by RT qPCR, and the experimental results were tested by S-W test. If the experimental results conform to the normal distribution, the T-test was used. If not, the Wilcoxon test was used. Spearman analysis was used to assess the relationship between miRNA and clinical characteristics. The relationship between miRNA and ischemic stroke was analyzed

by binary logistic regression method.

3. Result

3.1 Chip data characteristics

After searching geo database, we got miRNA microarray datasets GSE95204 and GSE86291, gene microarray datasets GSE58294 and GSE102541. The data set characteristics are shown in Table 1.

Table 1. Chip data characteristics

Accession Number	Platform ID	miRNA/mRNA	Numbers of HAVI samples	Numbers of HVT
GSE95204	GPL18058	miRNA	3	3
GSE86291	GPL18402	miRNA	7	4
GSE58294	GPL570	mRNA	23	23
GSE102541	GPL22755	mRNA	6	3

3.2 Chip data analysis results

The results of unqualified samples were removed through R software analysis. The threshold value of $P < 0.05$ and fold changes (FCs) > 1.2 was used to screen differential miRNAs. The results were as follows: 166 DEMs were screened by GSE95204, including 71 up-regulated miRNAs and 95 down-regulated miRNAs (Fig 1A). Seventy four DEMs were screened by GSE86291, including 30 up-regulated miRNAs and 44 down-regulated miRNAs (Fig 1B). Using $P < 0.05$, FCs > 1.2 as threshold to screen GSE58294 DEGs, 7293 DEGs were screened, among which 653 genes were up-regulated and 1167 genes were down-regulated (Fig 1C). Using $P < 0.05$, FCs > 1.5 as the threshold to screen GSE102541 DEGs, 5787 differential genes were screened, among which 2759 genes were up-regulated and 3028 genes were down-regulated (Fig 1D).

Fig 1A. Volcano plot of GSE95204

Fig 1B. Volcano plot of GSE86291

Fig 1C. Volcano plot of GSE58294

Fig 1D. Volcano plot of GSE102541

Through the comparative analysis of the data results, we found that there were 1107 DEGs overlapped(Fig 2A) and 5 DEMs overlapped(Fig 2B), and the 5 DEMs were all down-regulated(Table 2), among which the differential expression of hsa-mir-3156-5p was more significant in the two data sets, so we further analyzed hsa-mir-3156-5p in the follow-up experiment.

Fig 2A. Overlapping DEMs

Fig 2B. Overlapping DEGs

Table 2. Five overlapping miRNAs between GSE86291 and GSE95204

Overlapping DEMs	FCs in GSE95204	FCs in GSE86291	Characteristic
hsa-miR-3156-5p	-2.208	-2.871	down-regulated
hsa-miR-3137	-2.775	-0.424	down-regulated
hsa-miR-187-5p	-2.122	-0.406	down-regulated
hsa-miR-4688	-1.039	-0.391	down-regulated
hsa-miR-4738-3p	-1.328	-0.390	down-regulated

3.3 GO and KEGG pathway enrichment analysis of DEGs

Using webGestalt database, 1107 differential genes were analyzed by GO and KEGG. Among them, the top 10 enrichment results were shown in Fig 3A, Fig 3B, Fig 3C, and Fig 3D. The results showed that biological processes(BP) in GO analysis was mainly enriched in B cell homeostasis, immune response, cell activation, alpha-beta T cell proliferation, granulocyte differentiation, lymphocyte homeostasis, glucose 6-phosphate metabolic process, leukocyte homeostasis, embryonic skeletal system development and leukocyte activation. Cell component(CC) was mainly enriched in ficolin-1-rich granule, side of membrane, Golgi membrane, secretory granule, vacuole, Golgi apparatus, cytoplasmic vesicle part, mitochondrion, whole membrane and neuron part. Molecular function(MF) was mainly enriched in angiotensin receptor binding, galactosidase activity, type 1 angiotensin receptor binding, misfolded protein binding, C-C chemokine receptor activity, C-C chemokine binding, G protein-coupled chemoattractant receptor activity, chemokine receptor activity, chemokine binding and heat shock protein binding. The top 10 pathways of KEGG pathway were screened by count value. After manually removing other disease pathways, it was found that the top 10 pathways mainly concentrated in Metabolic

pathways, MAPK signaling pathway, Endocytosis, Chemokine signaling pathway, Cytokine-cytokine receptor interaction, Focal adhesion, Protein processing in endoplasmic reticulum, Regulation of actin cytoskeleton, Neuroactive ligand-receptor interaction, T cell receptor signaling pathway.

Fig 3A. BP of DEGs

Fig 3B. CC of DEGs

Fig 3C. MF of DEGs

Fig 3D. KEGG of DEGs

3.4 Construction of PPI network and screening of Hub gene

The PPI network of DEGs is constructed by using String database. The separated nodes and partially loosely connected nodes are removed. The remaining DEGs forms a complex multi center interaction network graph, which is visualized by Cytoscape software. Using the Cytohubba plug-in to analyze the PPI network and select the

nodes with high degree of interaction, and select the top 5%, that is, the top 40 hub genes(Fig 4A). Then, the Mcode plug-in is used to select the functional modules with $k\text{-score} > 12$ in PPI network, and two key modules are obtained. Key module 1 contains 16 nodes and 120 edges, and key module 2 contains 15 nodes and 105 edges (Fig 4b). Then we performed GO and KEGG analysis on these 40 Hub genes and two functional modules, screened enrichment results with $P < 0.05$ as the threshold, and manually eliminated other disease-related pathways of KEGG analysis. The results are shown in Fig 4C, Fig 4D, Fig 4E and Table 3.

Fig 4A. 40 Hub genes

Fig 4b. The key module 1(pink color) and key module 2(blue color) in PPI

Fig 4C. Go analysis of Hub gene.

(MF:red column, CC:green column , BP:blue column.)

Fig 4D. Go analysis of key module 1.

(MF:red column, CC:green column , BP:blue column.)

Fig 4E. Go analysis of key module 1.

(MF:red column, CC:green column , BP:blue column.)

Table 3. KEGG analysis for Hub, module 1 and module 2

Category	Description	P value	Count
Hub	Chemokine signaling pathway	<0.01	7
	Th17 cell differentiation	<0.01	4
	Inflammatory mediator regulation of TRP channels	<0.01	3
	Platelet activation	0.02	3
	Ubiquitin mediated proteolysis	0.02	3
	Cytokine-cytokine receptor interaction	0.03	4
	MAPK signaling pathway	0.04	4
	B cell receptor signaling pathway	0.04	2
Module 1	Chemokine signaling pathway	<0.01	6
	Neuroactive ligand-receptor interaction	<0.01	6
	Cytokine-cytokine receptor interaction	<0.01	5
Module 2	Sphingolipid signaling pathway	<0.01	3
	Ubiquitin mediated proteolysis	<0.01	6
	Protein processing in endoplasmic reticulum	<0.01	2

3.5 Target gene prediction and interaction analysis of DEMs

The target genes of five differential miRNAs were predicted using the three online databases described above. The results showed that there were 196 overlapping genes of mir-3156-5p, 191 overlapping genes of mir-3137, 182 overlapping genes of mir-187-5p, 137 overlapping genes of mir-4688 and 474 overlapping genes of mir-4738-3p(Fig. 5).

Fig 5. Target gene of DEMs

hsa-miR-4738-3p

We crossed these 1180 overlapping target genes with 1107 mRNAs to obtain 47 overlapping genes, and then crossed these 47 overlapping target genes with 40 hub genes to obtain three key genes: PTEN, lcp2 and SIAH1. The above results are shown in Table 4. The miRNA-gene regulatory network of 47 overlapping genes and corresponding miRNA is plotted using Cytoscape, as shown in Fig 6.

Table 4. List of overlapping genes, Hub genes and key genes

Overlapping genes	SMS, LRP2BP, GGCX, AUTS2, KPNA4, FAM118B, PEG3, VAV3, GPM6A, TMEM132C, PPP1R26, RORB, TRABD2B, ERCC1, LCP2, FAM32A, NFIB, RAG1, LINGO2, POU2F2, CD84, ZBTB47, CCDC62, ITGA5, SLC20A2, PUM2, ANKRD12, BEX4, CLN5, BCL7B, QKI, NECAB1, YWHAH, MEIS2, HAPLN1, ADAM12, NR3C2, MIER3, CCNYL1, CAMKV, PTEN, AFF1, RIMS2, THAP5, PAPPA, C9orf66, SIAH1
Hub genes	HRAS, PTEN, CCNB1, PKM, ARRB1, UBE2C, PA2G4, GNG2, UBE2D2, EIF4A3, SYK, IL1B, ACTR2, CCR2, FBXL19, S1PR4, LDHA, HIF1A, FOXP3, HIST1H2BA, LCP2, PRKACG, CCNF, BDKRB2, SMARCA4, HSP90AB1, FTSJ3, SIAH1, CDC6, WDR46, CCR3, CCR9, NMUR1, GATA1, HK1, HIST1H2BD, ARF1, HJURP, ATP5B, VPRBP
Key genes	PTEN, LCP2, SIAH1

Fig 6. miRNA-mRNA regulatory network

3.6 The level of miR-3156-5p in peripheral plasma of HAcI

In our experiment, we collected the peripheral plasma of HAcI patients and HVT. The relative expression of miR-3156-5p in plasma was determined by RT-qPCR. Since the sample size of HAcI group and HVT group were all less than 50, S-W test was used. The results showed that HAcI group and HVT group did not show any significant difference ($p>0.05$), which means that they accepted the original hypothesis (The original hypothesis: the data was normal distribution). The data of the two groups were compared by independent sample T-test. Finally, it was concluded that there was a significant difference in the level of miR-3156-5p expression between HAcI group and HVT group, and the specific results were shown in Figure 6. The primer sequences of internal reference and miR-3156-5p are shown in Table 4.

Table 4. The primer sequence of internal reference and miR-3156-5p

Primer name	primer sequence	Size
miR-3156-5p	CGCAAAGATCTGGAAGTGGAGACA	24
miR-423-5p (internal reference)	TGAGGGGCAGAGAGCGAGACTTT	23

Fig 6. Expression of miR-3156-5p in HAI and HVT

3.7 Relationship between miR-3156-5p and clinical characteristics

In order to further study the relationship between miR-3156-5p and ischemic stroke, we conducted Spearman correlation analysis to assess whether miR-3156-5p expression level in case group is related to traditional risk factors and laboratory indexes of ischemic stroke(Table 6). The basic characteristics of stroke group and control group are shown in Table 5. The indicators included age, gender, past medical history, smoking and drinking history, and related laboratory indexes.

Table 5. Basic characteristics of HAI group and HVT group

Clinical indicators	HAI (n=28)	HVT(n=20)	P
Gender (male / female)	male (71.4%)	male (35%)	0.011
	female (28.6%)	female (65%)	
Age (years)	63.679±12.347	60.400±12.313	0.369
Hypertension (n,%)	16(57.14)	11(55)	0.888
Diabetes (n,%)	5(17.86)	3(15)	0.796
Smoking (n,%)	11 (39.28)	4 (20)	0.161
Drinking (n,%)	6(21.43)	2(10)	0.306
Fasting blood glucose (mmol / L)	6.573±2.073	5.907±1.694	0.256
Stroke history (n,%)	4 (14.29)	3 (15)	0.947

Table 6. Correlation between the miR-3156-5p and clinical indicators

Clinical indicators	Correlation coefficient	P
Age (years)	-0.157	0.287
Gender (male / female)	-0.126	0.394
Hypertension (n,%)	0.147	0.319
Diabetes (n,%)	0.061	0.683
Smoking (n,%)	-0.144	0.328
Drinking (n,%)	-0.061	0.683
Stroke history (n,%)	-0.053	0.719
Fasting blood glucose (mmol / L)	-0.051	0.744
Blood homocysteine (μ mol/L)	0.189	0.207
NIHSS score	0.088	0.655
Cerebral infarction volume (cm ³)	0.029	0.905
Fibrinogen (g / L)	0.241	0.099
Total cholesterol (mmol / L)	0.101	0.506
Triglyceride (mmol / L)	0.373	0.011**
Low density lipoprotein (mmol / L)	0.060	0.693

Binary logistic regression was used to analyze whether miR-3156-5p could be used as a predictor of cerebral infarction(Table 7). After controlling for gender factors, the results are shown in the Table 8.

Table 7. Correlation between mir-3156-5p and stroke

	Regression coefficient	P	OR value	95% CI of OR
miR-3156-5p	-0.593	0.006	0.553	0.363 ~ 0.842

Table 8. Correlation between mir-3156-5p and stroke after controlling for gender

	Tier 1				Tier 2			
	B	SE	t	P	B	SE	t	P
Constant	0.021	0.224	0.095	0.925	3.500	1.159	3.021	0.004
Gender	0.360	0.137	2.634	0.011	0.302	0.127	2.374	0.022
miR-3156-5p					-0.107	0.035	-3.051	0.004
R ²		0.131				0.280		
Adj R ²		0.112				0.248		
F		F (1,46)=6.938,p=0.011				F (2,45)=8.752,p=0.001		
ΔR^2		0.131				0.149		
ΔF		F (1,46)=6.938,p=0.011				F (1,45)=9.311,p=0.004		

4. Discussion

Ischemic stroke refers to localized ischemic necrosis due to circulatory disturbance of the brain. MiRNA is an important post-transcriptional regulatory factor, showing different expression lineages at different stages of disease. As a hot research topic in recent years, miRNA has more research on cancer and autoimmune inflammatory diseases. Ischemic stroke is a common disease in the Department of Neurology. Most of the researches on ischemic stroke are aimed at treatment and secondary prevention. There are few basic researches on pathophysiology, especially in the hyperacute stage of ischemic stroke. In this study, miRNA and mRNA microarray data of hyperacute ischemic stroke were analyzed, and preliminary clinical verification was carried out, hoping to further understand the initiation process of ischemic stroke. In our study, we identified 1107 DEGs by analyzing the microarray data of HSCI patients. GO and KEGG analysis revealed that these genes were mainly involved in inflammatory factors activation, chemokine signaling pathway, immune response, cytokine receptor interaction, leukocyte homeostasis and activation. Then we constructed the PPI network of DEGs, and excavated the Hub genes and key modules, and further analyzed their biological functions and pathways. The results showed that the Hub genes and key module 1 were involved in chemokine, cytokine

pathway and inflammatory factor activation pathway. Studies have shown that after the occurrence of IS, microglia in the brain are activated and released a large number of inflammatory mediators, such as cytokines(IL-1 β , IL-6, TNF- α) and chemokines(MCP-1 and CXCL-1)[28], inducing inflammatory cascade. Neuroinflammation in the acute stage of IS will eventually lead to the destruction of BBB and induce neuron death[29]. The key module 2 gene is mainly associated with ubiquitin mediated proteolysis. In IS, ubiquitin reaction occurs immediately in neurons in the ischemic area and around the necrotic area. Studies have shown that the increased level of ubiquitin protein conjugates plays an important role in neuroprotection[30].

In this study, five kinds of DEMs were obtained by differential analysis, and the expression levels were all down-regulated. After screening, 47 pairs of miRNA-mRNA combinations were obtained. After crossing with Hub gene, three key genes were obtained, which were PTEN(phosphatase and tensin homolog), LCP2(lymphocytic cytosolic protein 2) and SIAH1(Siah E3 ubiquitin protein ligase 1). The expression levels were all down-regulated.

PTEN, located on chromosome 10q23.3, is a phospholipase composed of 403 amino acids. It is an important negative regulator in PI3K/Akt/mTOR pathway. PTEN can dephosphorylate PIP3 into PIP2 and antagonize PI3K/Akt pathway leading to cell death[31]. In Miao's experiment, it was proved that high expression of PTEN promoted neuronal cell death. Current studies have shown that PTEN plays an important role in ischemic stroke, affecting the treatment and prognosis of the disease[32]. The expression level of PTEN and phosphorylated PTEN in the ischemic core area and ischemic penumbra region was changed. Akt was up-regulated in the hippocampus and cortex of penumbra and extensive ischemic injury, suggesting that the down regulation of PTEN and the up regulation of Akt play a protective role in ischemic stroke[33]. The ubiquitination of PTEN protein will lead to neuronal death in IS[34].

LCP2 is an adapter protein-encoding gene that is involved in T cell antigen receptor (TCR)-activated protein tyrosine kinase pathway and is essential for normal T-cell development and activation by serine phosphorylation[35,36]. LCP2 mediates the second signal of CD28/ B7 family and regulates TCR signaling pathway[37]. In addition to T cells, there are also some studies that detail the function of LCP2 in other immune cells. LCP2 plays an important role in NK-cell mediated recognition of missing-self targets[38] and positively regulates antigen-induced mast cell activation by recruiting BCR[39]. The reduction of LCP2 may lead to immune dysfunction and excessive production of proinflammatory cytokines and auto antibodies[40]. Studies

have shown that LCP2 is deeply involved in the immune response through the regulation of immune cells. The function of LCP2 may vary from disease to disease, and further research is needed according to the gene expression changes of immune cells in IS.

SIAH1 protein is a E3 linked enzyme that recognizes substrate proteins and degrades them through ubiquitin protease system. SIAH has a wide variety of substrate proteins, which can participate in various tissue signal transduction pathways, and play an important role in cell cycle regulation, cell differentiation, apoptosis, tumor and neurodegenerative diseases. Many studies have shown that NO/GAPDH/Siah1 signaling pathway is the main way to mediate apoptosis. GAPDH is rich in sulfhydryl group, which can be oxidized by NO to form SNO-GAPDH. SIAH1 is a ubiquitin E3 ligase, which contains nuclear localization signal (NLS). SIAH1 can provide NLS to GAPDH enzyme protein, and promote the thiol nitrosylation of GAPDH enzyme protein under oxidative stress to produce SNO-GAPDH. The catalytic activity of SNO-GAPDH is decreased, and it can combine with SIAH1 and increase the stability of SIAH1, and form a complex (SNO-GAPDH/SIAH1) to transfer into the nucleus, which further leads to cell death or dysfunction[41,42]. After the occurrence of ischemic stroke, the imbalance between free radicals and antioxidant activities leads to oxidative stress, which may induce SNO/GAPDH/SIAH cell death cascade reaction and cause neuronal death. In addition, Lee's research has proved that SIAH1 plays a key role in the pathological process of Parkinson's disease[43]. It has been reported that SIAH1 is closely related to JNK. JNK can mediate neuronal apoptosis after spinal cord injury[44]. These evidences suggest that SIAH1 plays an important role in the nervous system.

We analyzed the gene function of 5 kinds of DEMs, and found that the target genes of these 5 kinds of DEMs were also mainly concentrated in chemokine signaling pathway, intercellular junction, platelet activation, B cell receptor signaling pathway, T cell receptor signaling pathway, which overlapped with the function of DEGs in the pathophysiological process of IS. After screening, 47 pairs of miRNA-mRNA combinations were obtained, which further suggested that these 5 miRNAs may be the participants in ischemic stroke thrombosis, early inflammation and subsequent cascade reactions.

In GSE86291 and GSE95204, the expression of miR-3156-5p was significant. Through further experimental verification, we proved that miR-3156-5p could be stably detected in plasma, and the plasma miR-3156-5p level of HAI patients was significantly lower than that of HVT ($P < 0.05$). After analyzing the expression level and clinical data of patients, it was found that there was a significant positive

correlation between the expression level and triglyceride level. Elevated triglyceride is one of the main characteristics of hyperlipidemia, and hyperlipidemia is the initiating factor of atherosclerosis. Existing studies have shown that in the development of hyperlipidemia, chronic inflammation can be triggered, and the activity and expression of various inflammatory factors increase [45]. A high level of miR-3156-5p may be involved in inhibiting the inflammatory process caused by hyperlipidemia.

The correlation between miR-3156-5p and stroke was further analyzed and evaluated by binary logistic regression. The results showed that: the regression coefficient of miR-3156-5p was -0.593, and showed a significant level of 0.01 ($z = -2.759$, $P = 0.006 < 0.01$), which means that mir-3156-5p will have a significant negative impact on the occurrence of stroke. The odds ratio (OR) was 0.553, which means that when miR-3156-5p was increased by one unit, the change (down-regulation) of stroke was 0.553 times. After using hierarchical regression to control the impact of gender factors, it can be seen from the Table 8 that there are two models involved in this hierarchical regression analysis. In model 1, the independent variable was gender, in model 2, mir-3156-5p was added to model 1, and the dependent variable was the occurrence of stroke. It can be seen from Table 8 that the R^2 value of the model is 0.131, which means that gender can explain 13.1% of the change of stroke. F-test was conducted on the model, and the results showed that the model passed F-test ($F = 6.938$, $P < 0.05$), which means that gender will definitely have an impact on stroke, and the model formula is: $\text{stroke} = 0.021 + 0.360 * \text{gender}$. The final specific analysis shows that: the regression coefficient of gender is 0.360, and it shows a significant ($t = 2.634$, $P = 0.011 < 0.05$), which means that gender will have a significant positive impact on stroke, that is, the incidence of stroke in men is higher than that in women. According to model 2: after adding miR-3156-5p analysis on the basis of model 1, the change of F value is significant ($p < 0.05$), which means that miR-3156-5p has explanatory significance to the model after its accession. In addition, the R^2 value increased from 0.131 to 0.280, which means that mir-3156-5p can explain 14.9% of stroke. Specifically, the regression coefficient of miR-3156-5p was -0.107, and showed a significant ($t = -3.051$, $P = 0.004 < 0.01$), which means that miR-3156-5p will have a significant negative impact on the occurrence of stroke.

In the miRNA-mRNA interactive network, we get 7 target overlapping genes of miR-3156-5p, including SMS, LRP2BP, GG CX, AUTS2, KPNA4, FAM118B and PEG3. Among the seven overlapping genes, Spermine Synthase (SMS) is involved in the synthesis of polyamines[46]. Polyamines have been shown to be essential for adipocyte differentiation[47,48,49]. In animal experiments, polyamine was proved to

limit the accumulation of fat in organisms[50,51]. When the expression of SMS decreases, especially in adult adipose tissue, triglyceride accumulation occurs[52]. The miR-3156-5p/SMS/Polyamines pathway may be the cause of triglyceride accumulation in HAI patients. The loss of SMS leads to neuronal degeneration due to the impairment of autophagy flux and mitochondrial function[53]. Gamma-glutamyl carboxylase(GGCX) is indispensable for the synthesis of mature vitamin K-dependent proteins, such as coagulation factors II, VII, IX, and X (FII, FVI, FIX, and FX, respectively) and anti coagulation factors such as protein C and protein S[54]. The AUTS2 gene plays an important role in regulation of neuronal migration, and its mutation is associated with mental disability, autistic characteristics, epilepsy and other neurological and somatic diseases[55]. Karyopherin subunit alpha 4(KPNA4), paternally expressed 3(PEG3) and family with sequence similarity 118 member B(FAM118B) have been verified to be oncogene in many cancers[56,57,58,59]. Low density lipoprotein receptor-related protein 2 binding protein(LRP2BP), as a new type of adaptor molecule binding to (LRP2), may recruit various protein molecules to form protein complexes by interacting with receptors, and mediate receptors to participate in signal transduction. At present, there are few studies on lrp2bp, and there are no reports on the relationship between lrp2bp and stroke[60].

Some studies have analyzed the function of miR-3156-5p[61], and found that the target genes of miR-3156-5p are mainly concentrated in transcription regulation, DNA template(CD86) and Toll like receptor 3(TLR3) pathway. Pathway analysis focused on neurotrophin signaling pathway, TGF- β Signal pathway(inhibition of microglia activation). In Rasheed's study[62], hyperlipidemia can directly or indirectly activate TLR system, leading to metabolic inflammation. In the adipose tissue of obese patients, the increased expression of TLR was found to be parallel to the increased expression of CD68, CD86 and CD163, the surface markers of monocytes / macrophages. CD68 and CD86 are markers of M1 macrophages, which indicates that hyperlipidemia may be a positive regulator of monocyte / macrophage aggregation in adipose tissue. In Asmaa's animal experiments[63], the expression of CD86 in the spleen of rats fed with high-fat diet was up-regulated, and CD86 was positively correlated with pro-inflammatory cytokines in adipose tissue. The existing research evidence shows that the formation and development of hyperlipidemia can cause inflammatory reaction, at the same time, the activity and expression of a variety of inflammatory factors are enhanced[64]. In peripheral macrophages, miR-3156-5p has a negative regulatory effect on CD86[35], and then participates in anti-inflammatory TLR3 signaling pathway. Combined with the above evidence, we

may infer that the expression level of miR-3156-5p is up-regulated with the increase of triglyceride level, which is involved in inhibiting the inflammatory process caused by hyperlipidemia.

The resident macrophages in brain are called microglia. They are in resting state (M0 phenotype) under physiological conditions, and rapidly activate under pathological conditions, transforming into pro-inflammatory M1 or anti-inflammatory M2 state. The expression of cell surface markers CD16 / 32, CD86 and CD40 increased in M1 state. The expression of CD86 was up-regulated in peripheral macrophages, which promoted the expression of TNF- α and IL- β [65,66,67]. Therefore, we can hypothesize that the low level expression of miR-3156-5p in HSCI patients may be one of the initiating factors of neuroinflammation induced by IS. The evidence also includes that TLR3 is mainly expressed in microglia in the brain, which can promote the survival of neurons after ischemia [68-69]. If we further explore the relationship between miR-3156-5p/CD86/TLR3 and neuroinflammation, we may further understand the pathogenesis of IS and find the corresponding adjuvant treatment.

5. Conclusion

In our study, a series of DEGs and Dens were screened by analyzing GEO data sets. In addition, the low expression of miR-3156-5p in HSCI patients may be one of the initiating factors of neuroinflammation induced by IS.

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Declarations

- **Ethics approval and consent to participate**

Not applicable

- **Consent for publication**

Not applicable

- **Availability of data and materials**

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The authors declare that they have no competing interests.

- **Authors' contributions**

Qi Li carried out the work of biological information, participated in the sequence alignment and drafted the manuscript. Wei Qi participated in the data collection and statistics. Jia-Nan Yang participated in the design of the study and performed the statistical analysis. Lei Li conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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